

# Erythroid Anion Transporter Assembly Is Mediated by a Developmentally Regulated Recruitment onto a Preassembled Membrane Cytoskeleton

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**Abstract.** Analysis of the expression and assembly of the anion transporter by metabolic pulse-chase and steady-state protein and RNA measurements reveals that the extent of association of band 3 with the membrane cytoskeleton varies during chicken embryonic development. Pulse-chase studies have indicated that band 3 polypeptides do not associate with the membrane cytoskeleton until they have been transported to the plasma membrane. At this time, band 3 polypeptides are slowly recruited, over a period of hours, onto a preassembled membrane cytoskeletal network and the extent of this cytoskeletal assembly is developmentally regulated. Only 3% of the band 3 polypeptides are cytoskeletal-associated in 4-d erythroid cells vs. 93% in 10-d erythroid cells and 36% in 15-d erythroid cells. This observed variation appears to be regulated primarily at the level of recruitment onto the membrane cytoskeleton rather than by different transport kinetics to the membrane or differential turnover of the soluble and insoluble polypeptides and is not depen-

dent upon the lineage or stage of differentiation of the erythroid cells. Steady-state protein and RNA analyses indicate that the low levels of cytoskeletal band 3 very early in development most likely result from limiting amounts of ankyrin and protein 4.1, the membrane cytoskeletal binding sites for band 3. As embryonic development proceeds, ankyrin and protein 4.1 levels increase with a concurrent rise in the level of cytoskeletal band 3 until, on day 10 of development, virtually all of the band 3 polypeptides are cytoskeletal bound. After day 10, the levels of total and cytoskeletal band 3 decline, whereas ankyrin and protein 4.1 continue to accumulate until day 18, indicating that the cytoskeletal association of band 3 is not regulated solely by the availability of membrane cytoskeletal binding sites at later stages of development. Thus, multiple mechanisms appear to regulate the recruitment of band 3 onto the erythroid membrane cytoskeleton during chicken embryonic development.

THE anion transporter, also known as band 3, is the major integral membrane protein of mammalian and avian erythrocytes, and is composed of two functionally distinct domains (23, 41). The membrane-spanning region mediates the exchange of internal bicarbonate for external chloride by an electroneutral transport mechanism (26). In this way, the carbon dioxide that is generated in respiring tissues is carried to the lungs in the form of bicarbonate anions dissolved in the serum. The cytoplasmic domain of band 3 provides the membrane attachment site for the erythrocyte membrane cytoskeleton through its interaction with ankyrin (2, 30), protein 4.1 (37) and protein 4.2 (11). In addition to its interaction with the membrane cytoskeleton, band 3 also possesses high-affinity binding sites for hemoglobin (9) and the glycolytic enzymes aldolase (38) and glyceraldehyde-3-phosphate dehydrogenase (47), although the latter site is absent in the chicken band 3 molecule (23). The complete amino acid sequence of the murine erythrocyte band 3 polypeptide has been deduced recently by cDNA sequencing (27) and shown to be highly homologous with the regions of the human erythrocyte band 3 protein which had been sequenced

previously (6, 24, 31). Furthermore, cDNA clones for human nonerythroid band 3 polypeptides, which have been described in many tissues (12, 14, 25), have been sequenced and shown to possess extensive homology with the murine erythroid molecule in the transmembrane domain but little, if any, homology in the cytoplasmic domain (13). The highly conserved nature of the transmembrane region of band 3 suggests that its structure and orientation in the membrane are critical for its anion transport function. However, the divergence in sequence observed between the cytoplasmic domain of erythroid and nonerythroid band 3 polypeptides suggests that this region of the molecule performs functions unique to a specific cell or tissue type.

Previous studies with adult human erythrocyte membranes have demonstrated that the cytoplasmic domain of erythroid band 3 binds the peripheral membrane cytoskeleton via its interaction with ankyrin in a 1:1 molar ratio. Ankyrin in turn is bound to the cytoskeleton through interaction with the  $\beta$ -subunit of the spectrin heterodimer (2). However, only 10–15% of the  $1 \times 10^6$  band 3 molecules per erythroid cell are associated with the membrane cytoskeleton (2). This was

postulated to be due to the fact that all of the available binding sites for band 3 ( $\sim 10^5$  ankyrin polypeptides per erythroid cell) were saturated under these conditions. Furthermore, only 12% of the band 3 polypeptides in ankyrin-depleted human erythrocyte membranes have the capacity to rebinding ankyrin (19). This suggests the possibility that the cytoskeletal-associated band 3 polypeptides have undergone structural or conformational changes before or as a result of their association with the membrane cytoskeleton, thus providing an additional level at which band 3 assembly could be regulated. Band 3 has also been shown to competitively inhibit the association of protein 4.1 with spectrin (37), suggesting that band 3 can utilize alternative binding sites in the peripheral membrane cytoskeleton. Studies from this laboratory have indicated that the synthesis of band 3 commences later than the synthesis of the peripheral membrane cytoskeletal polypeptides in early chicken erythroid development (44). In addition, it was postulated that the availability of band 3 polypeptides limits the extent to which the peripheral components can assemble as well as their stability (29, 33). However, the mechanism of assembly of band 3 onto the membrane cytoskeleton during erythroid development *in vivo* is unknown.

Through pulse-chase studies we demonstrate here that chicken erythroid band 3 assembles onto the membrane cytoskeleton with much slower kinetics (hours vs. minutes) than those previously described for the peripheral membrane cytoskeletal polypeptides  $\alpha$ -spectrin,  $\beta$ -spectrin, ankyrin, and protein 4.1 (4, 33, 40). In addition, protein synthesis inhibition experiments under conditions where the assembly of newly synthesized peripheral membrane cytoskeletal components is rapidly halted indicate that erythroid band 3 assembly occurs through the recruitment of band 3 polypeptides onto a preassembled cytoskeletal network. The extent of this cytoskeletal association is developmentally regulated, being very low early in development ( $\sim 3\%$  of total band 3 polypeptides), increasing to greater than 90% by day 10 of embryonic development and decreasing thereafter. Similar values obtained for cytoskeletal band 3 by pulse-chase and steady-state analyses on various days of embryonic development indicate that the developmental regulation of cytoskeletal band 3 levels is operative primarily at the recruitment step rather than through differential turnover of cytoskeletal band 3 polypeptides. Steady-state analyses have also shown that the very low levels of cytoskeletal band 3 in early embryonic development are most likely the result of limiting amounts of ankyrin and protein 4.1, the membrane cytoskeletal binding sites for band 3. As development proceeds and ankyrin and protein 4.1 polypeptide levels increase, a similar rise is seen in the level of cytoskeletal band 3 until on day 10 of development essentially all of the band 3 polypeptides are cytoskeletal bound. This peak in cytoskeletal band 3 assembly coincides with the maximum level of total band 3 polypeptides observed during embryonic development. After day 10, additional factors must influence the cytoskeletal association of band 3 because total and cytoskeletal band 3 levels decline even though ankyrin and protein 4.1 levels continue to increase. Thus, maximum steady-state levels for band 3 are attained several days before those observed for these two peripheral membrane cytoskeletal polypeptides. Finally, steady-state RNA analyses indicate that the extent of accumulation of band 3, ankyrin, and protein 4.1 polypeptides

in early embryonic development is not regulated primarily at the level of mRNA abundance. However, the more rapid decline in band 3 RNA levels relative to ankyrin and protein 4.1 between days 10 and 21 of embryonic development may be responsible for or contribute to the decrease in total band 3 polypeptide levels after day 10 in development.

## Materials and Methods

### Gel Electrophoresis

SDS-PAGE was performed according to Laemmli (28) as previously modified (22).

### Affinity Purification of Band 3 Antibodies

Band 3 polypeptides were purified from adult chicken erythrocyte membranes as described previously (12). The purified band 3 polypeptides were conjugated to cyanogen bromide-activated Sepharose CL-4B beads and used to affinity purify band 3 antibodies from a previously characterized band 3 antiserum (12). Briefly, a 1:1,000 dilution of the band 3 antiserum in Tris-buffered saline (TBS)<sup>1</sup> was incubated with the band 3 conjugated Sepharose CL-4B beads by rotating overnight at 4°C. The beads were washed several times with TBS and the antibodies were eluted by incubation in 3 vol of 0.2 M glycine (pH 2.3) for 2 min on ice. The beads were pelleted and the supernatant was neutralized by the addition of 1 vol of 1 M Tris base and dialyzed against several changes of TBS. This band 3 antibody preparation was used directly for immunoprecipitation and immunoblotting analyses.

### Immunoblotting

Circulating erythroid cells were isolated from 4-, 8-, 10-, 12-, 15-, 18-, and 21-d-old chicken embryos as described previously (4). The cells were resuspended in minimal essential medium (MEM), and total cell numbers were determined through duplicate counts with a hemocytometer. A 10% suspension of the cells was lysed in 150 mM NaCl, 10 mM Tris (pH 7.5), 5 mM MgCl<sub>2</sub>, 2 mM EGTA, 6 mM  $\beta$ -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1% (wt/vol) Triton X-100 by incubating for 5 min on ice. The lysate was centrifuged for 5 min in an Eppendorf centrifuge and the detergent-soluble supernatant was removed and made 1 $\times$  with SDS gel sample buffer. The detergent-insoluble pellet was resuspended in 20 mM Tris (pH 7.5), 1% (wt/vol) NaCl, 5 mM EGTA, 5 mM EDTA, 0.1% (wt/vol) SDS, 1% (wt/vol) Triton X-100, and 1% (wt/vol) sodium deoxycholate and sonicated twice for 30 s each. The sonicate was centrifuged for 10 min in an Eppendorf centrifuge and the insoluble residue was discarded. The detergent-insoluble fraction was made 1 $\times$  with SDS gel sample buffer and 1  $\times$  10<sup>6</sup> cell equivalents of both the detergent-soluble and detergent-insoluble fractions were electrophoresed on a 7.5% SDS polyacrylamide gel. These samples were transferred to nitrocellulose essentially according to the method of Towbin et al. (42), except transfer buffers contained 0.05% (wt/vol) SDS. The nitrocellulose filters were blocked by incubation for 2 h at 37°C in TBS that contained 0.25% gelatin (wt/vol). The filters were then incubated overnight at room temperature with either affinity-purified band 3 antibodies, ankyrin antisera (36), or protein 4.1 antisera (17) diluted 1:1,000 in TBS that contained 0.25% gelatin. The filters were washed in TBS containing 0.05% (vol/vol) Tween 20 and then incubated with <sup>125</sup>I-protein A in TBS that contained 0.25% gelatin and 0.05% Tween 20 for 30 min at room temperature. The protein A was iodinated using the reagent Iodogen (Pierce Chemical Co., Rockford, IL). Finally, the filters were washed in TBS containing 0.05% Tween 20, air-dried, and exposed to Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY) without using an intensifying screen at -80°C.

### Determination of Steady-State Level of Cytoskeletal Band 3 in Adult Chicken Erythroid Cells

Circulating erythroid cells were isolated from a 6-mo-old chicken and washed once in MEM as described previously (4). The cells were resuspended in 10 mM Tris (pH 7.5), 5 mM EDTA, 0.1 mM dithiothreitol, and

1. Abbreviations used in this paper: endo D or H, endoglycosidase D or H; TBS, Tris-buffered saline.

0.5 mM phenylmethylsulfonyl fluoride and lysed by sonicating two times for 30 s each. The resulting lysate was layered over a solution of 40% (wt/vol) sucrose in the same buffer and centrifuged for 30 min at 4°C in an Eppendorf centrifuge. The membranes were collected from the 40% sucrose interface and washed two times in lysis buffer. The membranes were pelleted and resuspended in 150 mM NaCl, 10 mM Tris (pH 7.5), 5 mM MgCl<sub>2</sub>, 2 mM EGTA, 6 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1% (wt/vol) Triton X-100 and incubated on ice for 5 min. The suspension was centrifuged for 10 min in an Eppendorf centrifuge and the detergent-soluble supernatant and the detergent-insoluble pellet were made 1× with SDS gel sample buffer. Equivalent amounts of each fraction were analyzed by electrophoresis on a 7.5% SDS polyacrylamide gel and either stained with Coomassie Blue or processed for immunoblotting with affinity-purified band 3 antibodies as described above.

### Immunoprecipitation

Circulating erythroid cells from 4, 10, and 15-d-old chicken embryos were isolated as described previously (4) and washed once in methionine-free MEM containing 5% dialyzed fetal calf serum. After a 10% suspension of these cells was incubated in this medium for 15 min at 37°C, 0.2 mCi/ml [<sup>35</sup>S]methionine (1,200–1,400 Ci/mmol, Amersham Corp., Arlington Heights, IL) was added and the cells were incubated for an additional 10 min at 37°C. At this time, an aliquot of the cells was removed and processed for immunoprecipitation (time 0). The remainder of the cells were pelleted and resuspended in MEM containing 5% fetal calf serum, incubated at 37°C, and additional aliquots were removed at the appropriate times. In experiments with 4-d erythroid cells, 5% chick embryo extract from 4-d-old chicken embryos was included in the chase medium. At each time point, the cells were lysed by incubation in 150 mM NaCl, 10 mM Tris (pH 7.5), 5 mM MgCl<sub>2</sub>, 2 mM EGTA, 6 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1% (wt/vol) Triton X-100 for 5 min on ice. The lysate was centrifuged for 5 min in an Eppendorf centrifuge. The detergent-soluble supernatant was diluted 10-fold in 20 mM Tris (pH 7.5), 1% (wt/vol) NaCl, 5 mM EGTA, 5 mM EDTA, 0.1% (wt/vol) SDS, 1% (wt/vol) Triton X-100, and 1% (wt/vol) sodium deoxycholate. The detergent-insoluble pellet was resuspended in the same buffer and sonicated twice for 30 s each. The insoluble material was pelleted and discarded. Affinity-purified band 3 antibodies were added to both the detergent-soluble and detergent-insoluble fractions and incubated overnight either at 4 or 37°C with rotation. Immune complexes were isolated with protein A-agarose beads and analyzed on 7.5% SDS polyacrylamide gels. Fluorography was performed by incubating the gels for 1 h in Enhance (New England Nuclear, Boston, MA) followed by a 1-h H<sub>2</sub>O wash. The gels were then dried and exposed to Kodak XAR-5 X-ray film at –80°C.

### Endoglycosidase H and Endoglycosidase D Digestions of Band 3 Immunoprecipitates

Erythroid cells from 10-d-old chicken embryos were pulsed with [<sup>35</sup>S]methionine for 10 min and chased for 2 h as described above. At each time point, immunoprecipitates were prepared with affinity-purified band 3 antibodies and the immune complexes that were bound to protein A-agarose beads were pelleted and resuspended in 20 mM Tris (pH 7.5) and 20 mM NaCl. 5 mU of endoglycosidase H (endo H) or 5 mU of endoglycosidase D (endo D) (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to half of this solution, while the other half contained buffer alone. These samples were incubated overnight either at 4°C or 37°C with rotation with identical results. The protein A-agarose beads were then washed, and the immune complexes were released in 1× SDS gel sample buffer and electrophoresed on a 7.5% SDS polyacrylamide gel. The gels were processed for fluorography and exposed to Kodak XAR-5 X-ray film as described above.

### Proteinase K Treatment of Intact Cells

Erythroid cells from 10- or 15-d-old chicken embryos were pulsed with [<sup>35</sup>S]methionine and chased for 2 h as described above. At each time point the cells were pelleted and resuspended in 5 mM Hepes (pH 7.1) and 155 mM choline chloride at 4°C. 20 mU of proteinase K conjugated to cellulose beads (EM Science, Darmstadt, Federal Republic of Germany) was added to half of the cells, whereas the other half contained buffer alone. The cells were incubated for 30 min at 4°C with rotation after which the proteinase K-cellulose beads were removed by differential centrifugation. The cells were then lysed and the samples processed for immunoprecipitation with affinity-purified band 3 antibodies as described above.

### RNA Isolation

Circulating erythroid cells were isolated from 5-, 8-, 10-, 15-, and 21-d-old chicken embryos as described previously (4). The cells were resuspended in MEM and total cell numbers were determined through duplicate counts with a hemocytometer. The cells were then homogenized in a solution that contained 5 M guanidinium isothiocyanate, 50 mM Tris (pH 7.5), 50 mM EDTA, 5% (vol/vol) β-mercaptoethanol, and 3% (wt/vol) sodium lauryl sarcosine using a dounce homogenizer. The homogenates were layered over a cushion of 5.7 M CsCl and centrifuged as previously described (8). To monitor recovery of total RNA from these erythroid cells, 10,000 cpm of <sup>32</sup>P-labeled adenosine phosphoribosyl transferase transcripts synthesized *in vitro* using SP6 RNA polymerase (32) were added to each homogenate before centrifugation. The recovery of the <sup>32</sup>P-labeled adenosine phosphoribosyl transferase transcript was then used to correct the amount of total RNA per 10<sup>6</sup> erythroid cells on each day of development examined.

### RNA Blotting and Hybridization

2 × 10<sup>6</sup> cell equivalents (reflecting recovery) of total erythroid RNA from 5-, 8-, 10-, 15-, and 21-d erythroid cells were electrophoresed on denaturing agarose gels and transferred to nitrocellulose as described previously (8). cDNA probes for ankyrin, pA1-4 (34), and protein 4.1, pFP02a (35), were <sup>32</sup>P-labeled by nick-translation (39) and hybridized to nitrocellulose filters (8).

### S1 Nuclease Assays

A λgt11 erythroid cDNA library was screened using p3(9-1) chicken band 3 cDNA (12) as a probe. Additional band 3 cDNA clones were isolated that comprised virtually the entire chicken erythroid band 3 mRNA. Sequencing of these chicken erythroid band 3 cDNA clones has revealed extensive homology with the murine erythroid band 3 molecule (Cox, J. V., and E. Lazarides, manuscript in preparation). S1 nuclease assays with one of these band 3 cDNA clones, p3(3-1), and a protein 4.1 cDNA, pFP020 (35), were performed as described previously (3, 35). Briefly, a BglII-EcoRI fragment from clone p3(3-1), which corresponds to 1,120 bp of the 3' translated and 250 bp of the 3' untranslated region of the chicken erythroid band 3 RNA, was end-labeled with the large fragment of DNA polymerase I in the presence of [α-<sup>32</sup>P]dNTPs. A XhoI-EcoRI fragment of pFP020 was similarly end-labeled. The end-labeled band 3 and protein 4.1 cDNA fragments were hybridized with 2 × 10<sup>6</sup> cell equivalents of total erythroid RNA from 5-, 8-, 10-, 15-, and 21-d erythroid cells in 80% formamide, 40 mM Pipes, 0.4 mM NaCl, and 1 mM EDTA for 12 h at 55 and 50°C, respectively. Samples were digested with S1 nuclease (3) for 30 min at 30°C and the fragments were resolved on 7 M urea-5% polyacrylamide gels. These gels were exposed to Kodak XAR-5 X-ray film at –80°C with an intensifying screen.

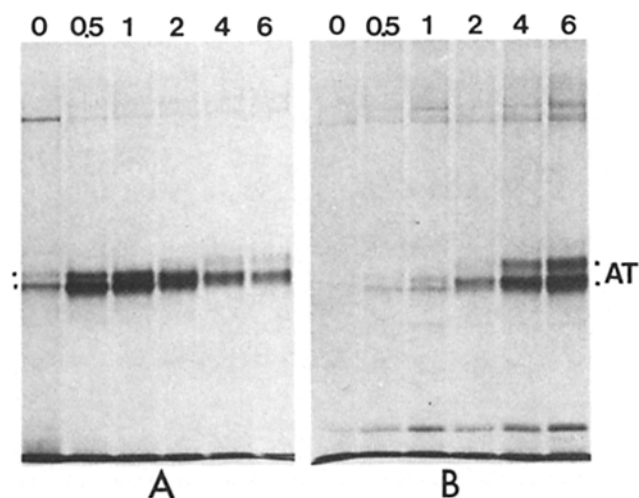
### Quantitation of Protein and RNA Levels

Autoradiograms of pulse-chase and steady state RNA and protein analyses were scanned with a Quick-Scan (Helena Laboratories, Beaumont, TX) and the area under the peaks were integrated using a Hewlett-Packard 3390A integrator (Hewlett-Packard Company, Avondale, PA).

### Results

#### Synthesis and Membrane-Cytoskeletal Assembly of the Anion Transporter in Erythroid Cells from 10-d-old Embryos

The fate of newly synthesized chicken erythroid anion transporter (band 3) polypeptides was examined by pulse-chase studies. Initial experiments were performed with erythroid cells from 10-d-old chicken embryos. The cells were pulsed with [<sup>35</sup>S]methionine for 10 min and chased in media containing unlabeled methionine for 6 h. At each time point the cells were lysed in an isotonic buffer containing 1% Triton X-100 and separated into a soluble supernatant and an insoluble pellet before immunoprecipitation with affinity-purified chicken erythroid band 3 antibodies. The autoradiogram in Fig. 1 illustrates that two polypeptides with apparent molec-

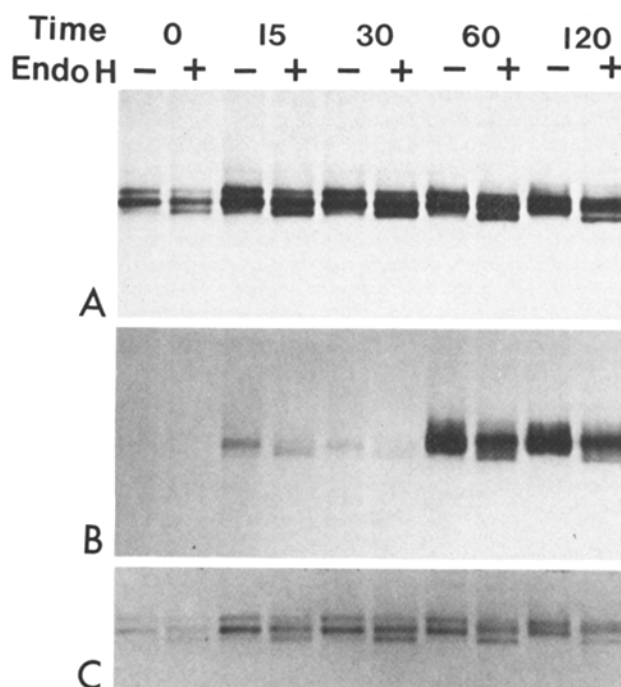


**Figure 1.** Synthesis and assembly of band 3 polypeptides in erythroid cells from 10-d-old chicken embryos. Erythroid cells from 10-d-old chicken embryos were incubated in methionine-free MEM that contained 5% dialyzed fetal calf serum and 0.2 mCi/ml [ $^{35}$ S]methionine for 10 min at 37°C. At the end of 10 min, an aliquot of cells was taken and lysed in isotonic buffer containing 1% Triton X-100. The lysate was separated into detergent-soluble and detergent-insoluble fractions by centrifugation. The remainder of the cells were pelleted and resuspended in MEM containing 5% fetal calf serum and incubated at 37°C. Additional aliquots were taken after 0.5, 1, 2, 4, and 6 h of chase in this medium and fractionated as described above. Immunoprecipitates were prepared from the (A) detergent-soluble and (B) detergent-insoluble fractions with affinity-purified band 3 antibodies and analyzed by electrophoresis on a 7.5% SDS polyacrylamide gel. AT, anion transporter (also referred to as band 3).

ular masses of 97 kD and 100 kD are detected, which are entirely detergent-soluble at the end of a 10-min pulse. An additional polypeptide of 95 kD was detected in the detergent-soluble fraction in some experiments at the end of a 10-min pulse (Figs. 2 A and 4 A). During the course of the chase, posttranslational modifications of these polypeptides eventually result in the 100- and 105-kD band 3 polypeptides (Fig. 1, A and B) that have been described previously in adult chicken erythroid membranes at steady state (12, 23). Furthermore, the newly synthesized band 3 polypeptides are gradually recruited from the soluble pool onto the detergent-insoluble cytoskeleton (Fig. 1 B). The association of band 3 with the cytoskeleton does not begin until ~30 min after the completion of synthesis (Fig. 1 B). This detergent-insoluble form of band 3 continues to accumulate until after 6 h of chase >70% of the band 3 polypeptides are associated with the cytoskeleton (Figs. 1 and 5 B).

The role of glycosylation in the mobility shifts observed for chicken erythroid band 3 in Fig. 1 has been investigated by a variety of enzymatic digestions. Erythroid cells from 10-d-old chicken embryos were labeled and processed as described above; however, before gel analysis one-half of each band 3 immunoprecipitate was digested with endo H which cleaves high-mannose chain sugars. As shown in Fig. 2, A and C, endo H treatment of the immunoprecipitate from the pulsed cells results in an additional detergent-soluble polypeptide of ~95 kD that is almost entirely absent in the undigested control. Essentially this same pattern of detergent-

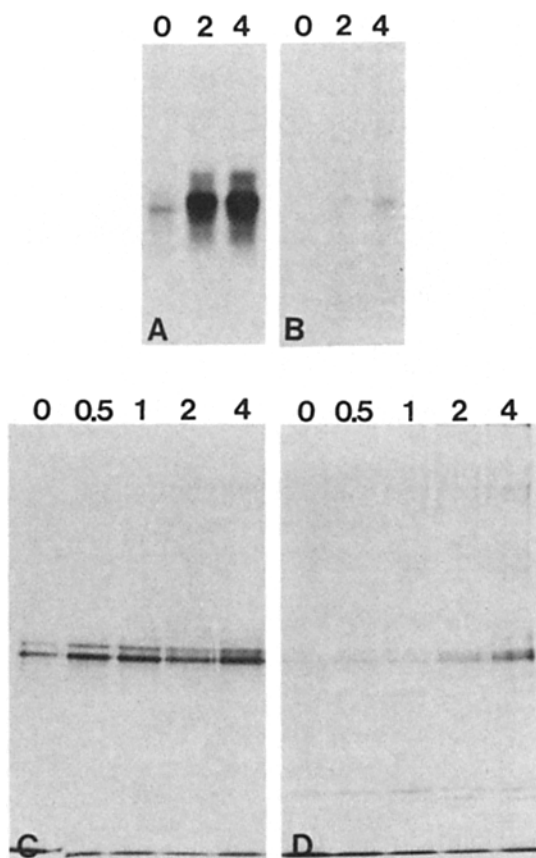
soluble band 3 polypeptides persists in both the control and digested samples up to 30 min of chase (Fig. 2, A and C) and this pattern is not altered by longer periods of digestion with endo H. Cytoskeletal-associated band 3 is first detected at low levels after 15 min of chase. In both the 15- and 30-min chase samples a single cytoskeletal polypeptide of 97 kD gives rise to a 95-kD polypeptide upon digestion with endo H (Fig. 2 B), indicating a precursor-product relationship between the two. Longer periods of chase result in a more diffuse pattern of band 3 polypeptides, some of which are insensitive to endo H digestion. However, digestion of the soluble polypeptides with endo H still results in a discrete band at 95 kD (Fig. 2, A and C), while digestion of the insoluble polypeptides yields a diffuse band that ranges from 95 to 97 kD (Fig. 2 B). Endo D digestion of identical samples to those described in Fig. 2 revealed that band 3 is not modified by the addition of N-linked complex sugars (data not shown). The modifications of band 3 that result in mobility shifts on SDS gels not accounted for by the addition of high-mannose chain sugars have yet to be identified.



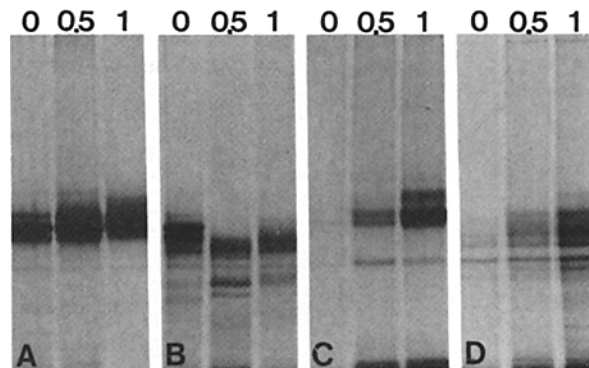
**Figure 2.** Endo H treatment of band 3 immunoprecipitates from 10-d erythroid cells. Erythroid cells from 10-d-old chicken embryos were labeled with [ $^{35}$ S]methionine for 10 min and chased for 15, 30, 60, and 120 min as described in Fig. 1. At each time point the cells were separated into (A and C) detergent-soluble and (B) detergent-insoluble fractions by lysis in an isotonic buffer containing 1% Triton X-100. Immunoprecipitates were prepared from each sample with affinity-purified band 3 antibodies and the immune complexes bound to protein A-agarose beads. One-half of the sample was digested with 5 mU of endo H overnight at 4°C while the other half was incubated in buffer alone. Immune complexes from the endo H-treated and control samples were released from the beads with 1× SDS gel sample buffer and resolved on a 7.5% SDS polyacrylamide gel. (C) A shorter exposure of the autoradiogram in A to more clearly illustrate the detergent-soluble polypeptides that are endo H-sensitive at each time point.

### **The Extent of Assembly of the Anion Transporter onto the Membrane Cytoskeleton Is Developmentally Regulated**

The extent of assembly of band 3 in erythroid cells from 10-d-old embryos is substantially higher than the steady-state level of cytoskeletal band 3 in adult chicken erythroid cells (>70% vs. 10–12%) as determined by scanning Coomassie Blue-stained gels and band 3 immunoblots (data not shown). This observation suggested that the extent of assembly of band 3 onto the membrane cytoskeleton is developmentally regulated. To analyze this possibility, erythroid band 3 assembly onto the membrane cytoskeleton was further investigated by examining erythroid cells from 4- and 15-d-old chicken embryos. The cells were labeled with [<sup>35</sup>S]methionine, chased, and fractionated as described above. The results of these studies indicate that the extent of band 3 assembly in 4- and 15-d erythroid cells differs from that seen in 10-d and adult erythroid cells. Only 6% of the newly synthesized band 3 polypeptides associate with the cytoskeleton during a 4-h chase (Fig. 3, *A* and *B*, and Fig. 5 *A*) in



**Figure 3.** Synthesis and assembly of band 3 polypeptides in day 4 and 15 erythroid cells. Erythroid cells from day 4 (*A* and *B*) and 15 chicken embryos (*C* and *D*) were labeled with [<sup>35</sup>S]methionine for 10 min and chased for 0.5, 1, 2, and 4 h as indicated and described in Fig. 1. At each time point the cells were lysed in an isotonic buffer containing 1% Triton X-100 and separated into a detergent-soluble (*A* and *C*) and a detergent-insoluble fraction (*B* and *D*). Immunoprecipitates were prepared from each sample using affinity-purified band 3 antibodies and the immune complexes were analyzed by electrophoresis on a 7.5% SDS polyacrylamide gel.

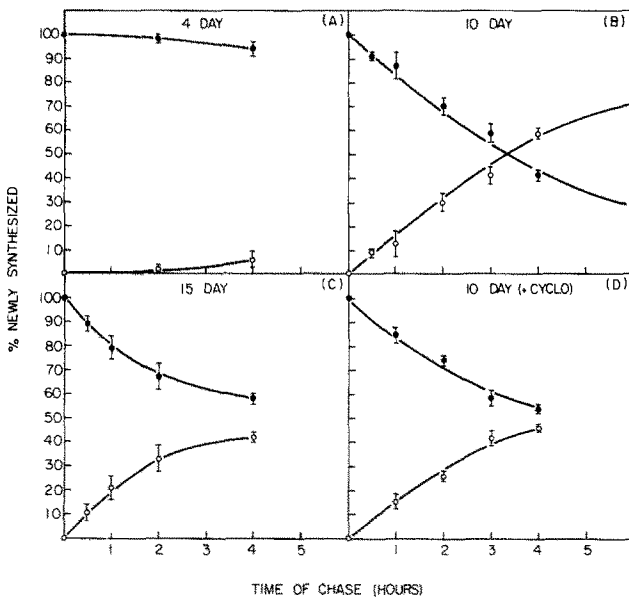


**Figure 4.** Extracellular proteinase K digestion of erythroid cells from 10-d-old chicken embryos. Erythroid cells from day 10 embryos were labeled with [<sup>35</sup>S]methionine for 10 min and chased for 0.5 and 1 h as described in Fig. 1. At each time point the cells were pelleted and resuspended in 5 mM Hepes (pH 7.1) and 155 mM choline chloride. 10 mU of proteinase K was added to one-half of the cells (*B* and *D*), whereas the other half contained buffer alone (*A* and *C*). The cells were incubated for 30 min at 4°C with rotation after which the proteinase K-cellulose beads were removed by differential centrifugation. The cells were separated into a detergent-soluble (*A* and *B*) and a detergent-insoluble fraction (*C* and *D*) by lysis in an isotonic buffer containing 1% Triton X-100. Immunoprecipitates were prepared from each sample with affinity-purified band 3 antibodies and analyzed by electrophoresis on a 7.5% SDS polyacrylamide gel.

4-d erythroid cells. After a similar chase period in 10-d erythroid cells, 59% of the newly synthesized band 3 polypeptides are cytoskeletal-associated (Fig. 5 *B*). The kinetics of assembly of band 3 in 15-d erythroid cells are similar to those seen in 10-d cells up to 2 h of chase (Fig. 3, *C* and *D*, and Fig. 5 *C*). However, at this time the level of cytoskeletal band 3 plateaus in 15-d erythroid cells at ~40% of the newly synthesized polypeptides, while cytoskeletal band 3 continues to accumulate in 10-d cells to a final value of ~70% (Fig. 5, *B* and *C*). Similar pulse-chase studies with 5-d erythroid cells resulted in 15% of the newly synthesized polypeptides associating with the membrane-cytoskeleton during a 4-h chase (data not shown). Although the mechanism regulating the association of band 3 with the cytoskeleton is not known, it is clear from these studies that the extent of band 3 assembly onto the membrane cytoskeleton varies dramatically during development and thus is under developmental control.

### **The Kinetics of Transport of Newly Synthesized Band 3 Polypeptides to the Plasma Membrane Do Not Vary As a Function of Development**

The possibility that the variation observed in the assembly of band 3 during development was due to different transport kinetics of the band 3 polypeptides to the plasma membrane was investigated by monitoring the sensitivity of the newly synthesized polypeptides to extracellular proteinase K digestion. Erythroid cells from 10-d chicken embryos were pulsed and chased as described above; however, before lysis in Triton X-100 containing buffers one-half of the cells from each time point was digested with proteinase K coupled to cellulose beads for 30 min at 4°C. The beads were removed



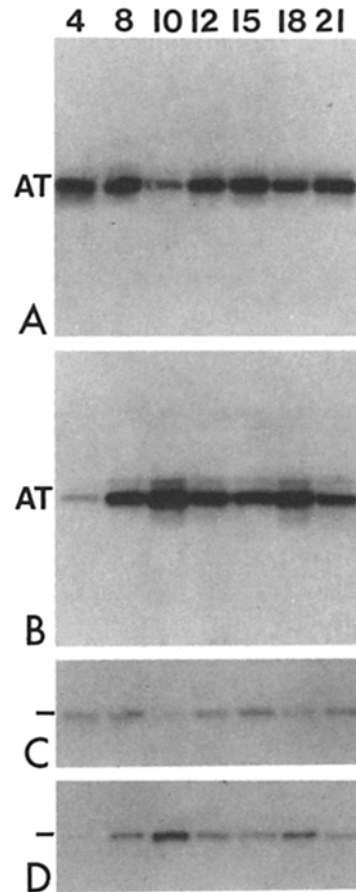
**Figure 5.** Synthesis and assembly of band 3 polypeptides in erythroid cells from various stages of embryonic development. The detergent-soluble (●) and the detergent-insoluble fractions (○) of [<sup>35</sup>S]methionine-labeled 4 (A), 10 (B), and 15-d erythroid cells (C) were immunoprecipitated with affinity-purified band 3 antibodies as described in Figs. 1 and 3. Erythroid cells from 10-d-old chicken embryos were also labeled with [<sup>35</sup>S]methionine for 10 min and chased for 30 min as described in Fig. 1. At this time cycloheximide was added to a final concentration of 2 μM and aliquots were removed after 1, 2, 3, and 4 h of total chase. The cells from each time point were lysed in an isotonic buffer containing 1% Triton X-100 and separated into a detergent-soluble (●) and a detergent-insoluble fraction (○). Immunoprecipitates were prepared from each sample using affinity-purified band 3 antibodies (D) and the immunoprecipitates were analyzed by electrophoresis on a 7.5% SDS polyacrylamide gel. The resulting autoradiograms from each pulse-chase experiment were scanned and the area under each peak was integrated. The 0 time point represents the time when cold methionine was added. The symbols represent the average of three experiments from 4-d cells, five experiments from 10-d cells, four experiments from 15-d cells, and two cycloheximide experiments. The error bars represent the standard deviation at each time point.

by differential centrifugation and the cells were lysed and processed for immunoprecipitation. At the end of a 10-min pulse, virtually all of the detergent-soluble band 3 polypeptides are protected from extracellular proteinase K digestion (Fig. 4, A and B). The detergent-soluble and detergent-insoluble polypeptides that are susceptible to proteinase K at the end of the pulse probably arise from a small fraction of the newly synthesized polypeptides undergoing transport to the plasma membrane during the 4°C proteinase K incubation (Fig. 4). After a 30-min chase, the band 3 polypeptides have associated with the membrane cytoskeleton to a greater extent (Fig. 4, A and C), and at this time the detergent-soluble (Fig. 4 B) and detergent-insoluble (Fig. 4 D) band 3 polypeptides are completely susceptible to extracellular proteinase K digestion. This indicates that most, if not all, of the band 3 polypeptides are transported to the plasma membrane before their association with the membrane cytoskeleton. Furthermore, essentially identical results obtained with erythroid cells from 15-d-old chicken embryos

(data not shown) suggest that the variation observed in the assembly of band 3 during embryonic development is not due to different transport kinetics of band 3 polypeptides to the plasma membrane.

### **Steady-State Levels of Total and Cytoskeletal Erythroid Band 3 Increase and Then Decline during Embryonic Development**

The pulse-chase studies described above have established that the fraction of newly synthesized band 3 polypeptides that associate with the membrane cytoskeleton varies during embryogenesis. This developmental difference in the extent of band 3 assembly observed in pulse-chase experiments



**Figure 6.** Steady-state levels of detergent-soluble and detergent-insoluble erythroid band 3 polypeptides during chicken embryonic development. Erythroid cells isolated from 4-, 8-, 10-, 12-, 15-, 18-, and 21-d chicken embryos were resuspended in MEM and total cell numbers were determined through duplicate counts with a hemocytometer. The cells were then pelleted and lysed in an isotonic buffer containing 1% Triton X-100. The lysate was separated into a detergent-soluble (A and C) and a detergent-insoluble fraction (B and D) by centrifugation.  $1 \times 10^6$  cell equivalents of each fraction were electrophoresed on a 7.5% SDS polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose filter was probed with affinity-purified band 3 antibodies and immunoreactive polypeptides were detected by incubation with <sup>125</sup>I-protein A followed by autoradiography. C and D are shorter exposures of the autoradiograms in A and B, respectively, to illustrate more clearly the variation in the levels of soluble and cytoskeletal band 3 during development. AT and bar, anion transporter.



could be regulated directly at the assembly step or regulated by additional factors such as the differential turnover of cytoskeletal-associated band 3. This issue was examined by comparing the kinetics of band 3 assembly to the steady-state levels of this polypeptide as determined by immunoblotting analysis. Erythroid cells from 4-, 8-, 10-, 12-, 15-, 18-, and 21-d-old chicken embryos were isolated and lysed in an isotonic buffer containing 1% Triton X-100. The lysate was separated into a soluble supernatant and an insoluble pellet and  $1 \times 10^6$  cell equivalents of each were electrophoresed on a SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with affinity-purified band 3 antibodies. The band 3 immunoblot (Fig. 6) shows that the steady-state levels of both total and cytoskeletal band 3 fluctuate considerably during embryonic development. The per-cell amount of total band 3 is lowest in 4-d erythroid cells and increases approximately fourfold to its peak value on day 10 (Fig. 6 and Table I). By day 12 the total amount of band 3 per cell has decreased by 50% and it remains essentially constant until hatching. The fraction of total band 3 that is cytoskeletal-associated is also lowest in 4-d erythroid cells where only 3% of the band 3 is insoluble (Fig. 6 and Table I). However, by day 10 >90% of the band 3 polypeptides are associated with the cytoskeleton. This value ranges from 32 to 68% on subsequent days of embryonic development (Table I). The similar levels of cytoskeletal band 3 obtained by pulse-chase (Fig. 5) and steady-state analyses (Table I) suggest that the amount of band 3 that associates with the cytoskeleton is primarily regulated at the assembly step. Furthermore, this step is developmentally controlled, reaching its highest level by day 10 of development and declining approximately threefold by hatching.

Additional experiments were performed to determine if the lineage of erythroid cells in early embryonic development has any effect on the association of band 3 with the membrane cytoskeleton. Erythroid cells from 4-d chicken embryos are derived entirely from cells of the primitive lineage, which are released early in development in a single burst and develop as a somewhat synchronous population. On day 5 of development, cells of the definitive lineage which will persist into adult life begin to enter the circulation (7). Erythroid cells from 7-d chicken embryos, which are composed of 50% definitive and 50% primitive lineage cells, were fractionated by centrifugal elutriation, which has been shown to separate cells of the two lineages on the basis of a difference in size (18). The elutriated cells were lysed and fractionated as described above and immunoblotting analysis of the detergent-soluble and insoluble fractions was performed with affinity-purified band 3 antibodies. Although the cells of the two lineages are at different stages of erythroid differentiation, the immunoblots indicated that their cytoskeletal band 3 levels are comparable (data not shown), suggesting that the environment of these cells is important in regulating the cytoskeletal association of band 3 polypeptides.

#### ***Band 3 Assembly onto the Membrane Cytoskeleton Occurs Independently of the Assembly of Peripheral Membrane Cytoskeletal Polypeptides***

Previous studies have indicated that the assembly of the peripheral membrane cytoskeletal polypeptides ankyrin,  $\alpha$ - and  $\beta$ -spectrin and protein 4.1 is extremely rapid and requires

**Table I. Quantitation of Protein Levels during Embryonic Development**

Day	AT		Ankyrin		Protein 4.1	
	% MAX*	% CSK†	% MAX*	% CSK†	% MAX*	% CSK†
4	26.9	2.6	7.7	75.5	3.9	84
8	74.4	53.4	16.7	89.3	33.2	>99
10	100	93.4	57.8	>99	69.7	>99
12	53.3	58	63.8	96.2	76.1	>99
15	55	35.7	49.1	89.7	65.4	>99
18	64.3	68	100	98.3	100	>99
21	49.3	32.3	60	91.8	82.8	97.9

The steady-state levels of detergent-soluble and detergent-insoluble band 3, ankyrin, and protein 4.1 polypeptides were determined in 4-, 8-, 10-, 12-, 15-, 18-, and 21-d erythroid cells as described in Figs. 5 and 6. The autoradiograms resulting from these immunoblots were scanned and the area under each peak was integrated. The total amount of band 3, ankyrin, and protein 4.1 polypeptides was calculated from the sum of the detergent-soluble and detergent-insoluble fractions. The values for detergent-soluble and detergent-insoluble protein 4.1 and ankyrin polypeptides in 4-d erythroid cells were obtained from longer exposures of the autoradiograms in Fig. 6. AT, anion transporter.

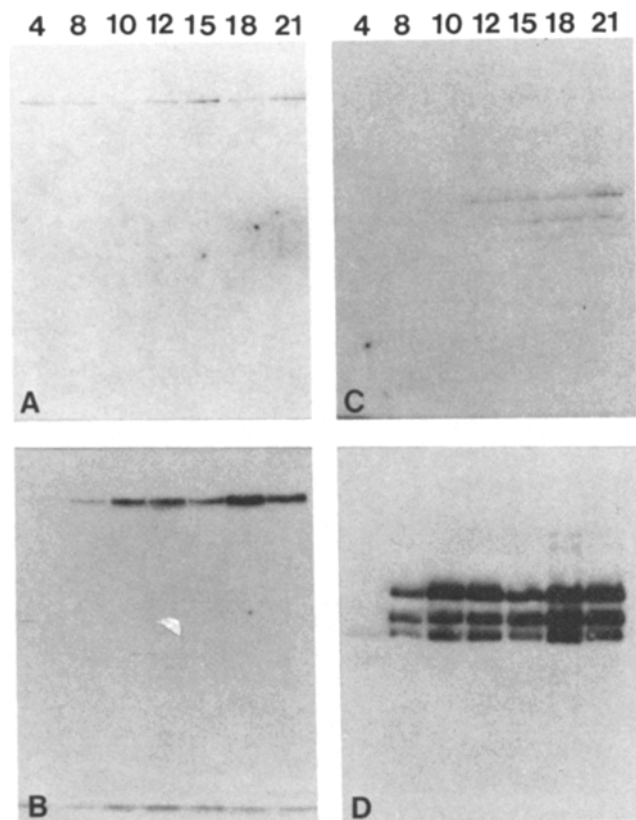
\* The % MAX values in the table represent the levels of these polypeptides relative to their peak value (100) during embryonic development.

† The % CSK values represent the fraction of these polypeptides that are cytoskeletal-associated from each day of development examined.

continuous protein synthesis (4, 33, 40, 45). Thus, the kinetics of assembly of band 3 presented here and those of the peripheral membrane cytoskeletal polypeptides are significantly different. In order to determine whether the association of band 3 with the membrane cytoskeleton also requires continuous protein synthesis and the availability of newly synthesized peripheral membrane cytoskeletal polypeptides the following experiments were performed. Erythroid cells from 10-d chicken embryos were pulsed with [ $^{35}$ S]methionine for 10 min and chased in media containing unlabeled methionine for 30 min to insure completion of nascent polypeptide chains. At this time the cells were split in half, and to one-half, 2  $\mu$ M cycloheximide was added. Aliquots were taken from both the cycloheximide-treated and control cells at various time points and the association of band 3 with the cytoskeleton was assayed by detergent insolubility. Fig. 5, *B* and *D* illustrate that the interaction of the newly synthesized band 3 polypeptides with the cytoskeleton is not inhibited by cycloheximide until 4 h of chase has elapsed. At this time 46% of the band 3 polypeptides have associated with the cytoskeleton in the cycloheximide-treated cells (Fig. 5 *D*) as compared with 59% in control cells (Fig. 5 *B*). This suggests that the binding site that links band 3 to the cytoskeleton is saturable. Because previous studies have shown that there is no soluble pool for  $\alpha$ -spectrin,  $\beta$ -spectrin, ankyrin, and protein 4.1 in erythroid cells which is competent to undergo assembly in the absence of protein synthesis (40, 46), these results suggest that the detergent insolubility of band 3 most likely arises through its interaction with a preassembled cytoskeletal network.

#### ***Steady-State Levels of Erythroid Ankyrin and Protein 4.1 Increase Continuously during Embryonic Development***

The band 3 immunoblotting analysis has indicated that cytoskeletal-associated band 3 levels are very low in erythroid cells from 4-d chicken embryos even though band 3 polypeptides are relatively abundant at this stage of development. This result suggests that the availability of components in the



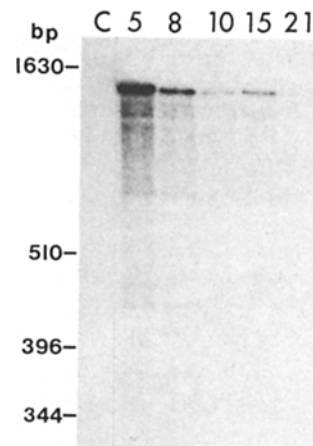
**Figure 7.** Steady-state levels of detergent-soluble and detergent-insoluble ankyrin and protein 4.1 polypeptides during chicken embryonic development.  $1 \times 10^6$  cell equivalents of the detergent-soluble (A and C) and the detergent-insoluble fraction (B and D) from 4-, 8-, 10-, 12-, 15-, 18-, and 21-d erythroid cells prepared as described in Fig. 5 were electrophoresed on 7.5% SDS polyacrylamide gels and transferred to nitrocellulose. The filters were probed with a 1:1,000 dilution of either anti-ankyrin antisera (A and B) or anti-protein 4.1 antisera (C and D) and the immunoreactive polypeptides were detected by incubation with  $^{125}$ I-protein A followed by autoradiography. In each case the exposure of the detergent-soluble fraction was four times longer than for the detergent-insoluble fraction.

erythroid membrane cytoskeleton may limit the cytoskeletal association of band 3 in 4-d erythroid cells. Previous investigators have demonstrated that ankyrin and protein 4.1 specifically interact with band 3 *in vitro* (2, 30, 37), suggesting that they may provide the binding sites that link band 3 with the membrane cytoskeleton. For this reason, the pattern of accumulation of ankyrin and protein 4.1 has also been examined throughout embryonic development by immunoblotting analyses to determine whether the availability of these polypeptides could limit the association of band 3 with the membrane cytoskeleton during development. Identical samples to those described in Fig. 6 were electrophoresed on SDS polyacrylamide gels, transferred to nitrocellulose and probed with either anti-ankyrin (Fig. 7, A and B) or anti-protein 4.1 antibodies (Fig. 7, C and D). The exposure of the autoradiograms in each case was four times longer for the detergent-soluble fraction (Fig. 7, A and C) than for the detergent-insoluble fraction (Fig. 7, B and D). The immunoblots illustrate that both ankyrin and protein 4.1, which are almost entirely detergent-insoluble at all days examined except day

4, accumulate in a similar manner during embryonic development (Fig. 7 and Table I). The abundance of each of these polypeptides is lowest in 4-d erythroid cells as was observed for band 3. However, the onset of ankyrin and protein 4.1 accumulation lags behind band 3 in early embryonic development (Table I). Furthermore, as the per cell amount of ankyrin and protein 4.1 begins to increase, a concurrent rise is seen in the level of cytoskeletal-associated band 3, until >90% of the band 3 polypeptides are detergent-insoluble on day 10 of development (Table I). This result suggests that the availability of the ankyrin and protein 4.1 polypeptides may limit the association of band 3 with the membrane cytoskeleton in very early development. After day 10 of embryonic development, the steady-state levels of cytoskeletal ankyrin and protein 4.1 continue to rise to their peak values on day 18 (Fig. 7, B and D and Table I), while at the same time cytoskeletal, as well as total, band 3 levels decline (Table I), suggesting that these polypeptides no longer limit the association of band 3 with the membrane cytoskeleton. The data presented in Table I also indicate that ankyrin and protein 4.1 can accumulate in the membrane cytoskeleton irrespective of the level of cytoskeletal band 3 (see Discussion).

#### **Steady-State RNA Levels for Erythroid Band 3, Ankyrin, and Protein 4.1 Decline Noncoordinately during Embryonic Development**

The steady-state immunoblotting analyses reported thus far have indicated that band 3 accumulates noncoordinately with respect to ankyrin and protein 4.1 during development. To determine whether this noncoordinate accumulation of these erythroid membrane cytoskeletal polypeptides is regulated at the level of mRNA abundance, quantitative S1 nuclease and RNA blot analyses were performed. For each assay  $2 \times 10^6$  cell equivalents of total cellular RNA from 5-, 8-, 10-, 15-, and 21-d erythroid cells were probed with cDNAs specific for band 3, ankyrin, or protein 4.1. The results of one S1 nuclease experiment using a band 3-specific cDNA are shown



**Figure 8.** Steady-state levels of erythroid band 3 RNA determined by quantitative S1 nuclease protection assays. Erythroid cells isolated from 5-, 8-, 10-, 15-, and 21-d chicken embryos were resuspended in MEM and total cell numbers were determined through duplicate counts with a hemocytometer. RNA was prepared from these cells as described in the Materials and Methods. A  $^{32}$ P-end-labeled BglII-EcoRI fragment from the erythroid band 3 cDNA clone, p3(3-1), was hybridized with  $2 \times 10^6$  cell equivalents of total eryth-

roid RNA from 5-, 8-, 10-, 15-, and 21-d erythroid cells for 12 h at 55°C. Samples were then digested with S1 nuclease for 30 min at 30°C and the fragments were resolved on a denaturing 7M urea-5% polyacrylamide gel. Lane C represents the no RNA control. Size markers in base pairs are several end-labeled restriction fragments of Hinf I-digested pBR322 DNA.



in Fig. 8. Band 3 RNA levels are highest in 5-d erythroid cells and decline rapidly on subsequent days of development, until they are virtually undetectable on day 21. Quantitation of this experiment has revealed that the decrease in band 3 RNA levels during development mimics the decline seen in total erythroid RNA (Table II). Similar analyses indicate that ankyrin and protein 4.1 RNA levels are also highest in 5-d erythroid cells and decrease during embryonic development although more gradually than band 3 and total RNA levels (Table II). The observed decrease in the per cell amount of band 3, ankyrin, and protein 4.1 RNA during development in conjunction with the severalfold increase in their polypeptide levels indicates that the extent of accumulation of these erythroid membrane cytoskeletal polypeptides is not determined primarily at the level of mRNA abundance early in development.

## Discussion

The mechanisms that regulate the association of chicken erythroid band 3 with the membrane cytoskeleton have been investigated on various days of embryonic development through pulse-chase and steady-state analyses. These studies have revealed that both the extent of accumulation and the cytoskeletal association of erythroid band 3 are developmentally regulated. Pulse-chase experiments have shown that the fraction of newly synthesized band 3 polypeptides that associate with the membrane cytoskeleton varies dramatically as a function of development. Only 6% of the newly synthesized band 3 polypeptides associate with the membrane cytoskeleton in erythroid cells from 4-d chicken embryos vs. 70% in 10-d erythroid cells and 40% in 15-d erythroid cells. Immunoblotting analysis has further demonstrated that the steady-state levels of cytoskeletal band 3 reflect the values for cytoskeletal band 3 obtained in pulse-chase studies. These results in conjunction with the protease digestion studies indicate that the extent of association of band 3 with the membrane cytoskeleton during development is primarily regulated at the assembly step rather than by differential turnover of the polypeptides or different transport kinetics to the plasma membrane.

The pulse-chase studies presented here also indicate that the assembly of band 3 onto the membrane cytoskeleton occurs with much slower kinetics than the assembly of the peripheral membrane cytoskeletal polypeptides  $\alpha$ -spectrin,  $\beta$ -spectrin, ankyrin, and protein 4.1 (hours vs. minutes; 4, 33, 40). Newly synthesized erythroid band 3 polypeptides do not begin to associate with the membrane cytoskeleton until ~30 min of chase has elapsed. By this time, the polypeptides have been quantitatively transported to the plasma membrane as monitored by their susceptibility to extracellular proteinase K digestion. These transport kinetics are similar to those previously described for murine erythroid band 3 (5). During longer periods of chase, the newly synthesized band 3 polypeptides slowly associate with the membrane cytoskeleton. At present, it is unclear why band 3 polypeptides in transit to the plasma membrane are unable to bind to the cytoskeleton in that they are presumably in an orientation that is accessible to cytoplasmic polypeptides, as has been shown for murine erythroid band 3 (5). One possibility is that the conformation of the polypeptides is such that they cannot interact with the membrane cytoskeletal binding sites.

**Table II. Quantitation of RNA Levels during Embryonic Development**

Day	Total RNA	AT RNA	Ankyrin RNA	Protein 4.1 RNA
		% MAX*	% MAX*	% MAX*
5	100	100	100	100
8	18.9	20.4	ND	50.7
10	8.3	6.8	57.5	31.8
15	5.3	11.7	64.7	38.9
21	3.0	<1	<1	17.1

The steady-state levels of erythroid band 3 RNA were determined in 5-, 8-, 10-, 15-, and 21-d erythroid cells by an S1 nuclease protection experiment as described in Fig. 7. The autoradiogram resulting from this experiment was scanned and the area under each peak was integrated. A similar S1 nuclease protection experiment was performed to determine the levels of protein 4.1 RNA as described in the Materials and Methods. This experiment indicated that there are multiple RNA variants for protein 4.1 and the abundance of each individual variant is developmentally regulated (38). Protein 4.1 RNA levels were also examined by RNA blotting as described in the Materials and Methods. A single band of 6.6 kb was detected on each day of development examined indicating that each of the protein 4.1 RNA variants is 6.6 kb in size (38). The autoradiograms resulting from the S1 nuclease experiment and the RNA blot analysis were scanned and the area under each peak was integrated. The relative amounts of protein 4.1 RNA obtained by these two techniques on the various days of embryonic development were essentially identical. Ankyrin RNA levels during embryonic development were also determined by RNA blotting analysis as described in the Materials and Methods. The autoradiogram resulting from this RNA blot was scanned and the area under each peak was integrated. Finally, total erythroid RNA levels were determined on each day of development examined as described in the Materials and Methods. AT, anion transporter.

\* The % MAX values in the table represent the levels of each RNA relative to their peak value (100) during embryonic development. ND, not determined.

Alternatively, the rapid kinetics of assembly onto the membrane cytoskeleton of the peripheral membrane cytoskeletal polypeptides may sequester all available binding sites in the vicinity of the plasma membrane. The possibility should also be considered that our assay for membrane-cytoskeletal association of band 3, namely insolubility in buffers containing Triton X-100, may not detect unstable complexes between band 3 and components of the membrane cytoskeleton.

The mechanism of assembly of band 3 polypeptides onto the membrane cytoskeleton subsequent to transport to the plasma membrane was further investigated through the use of inhibitors of protein synthesis. These experiments have shown that the assembly of newly synthesized band 3 polypeptides onto the membrane cytoskeleton is unaffected by cycloheximide until 4 h of chase have elapsed. By this time, cycloheximide-treated cells have assembled 46% of their newly synthesized band 3 polypeptides whereas untreated controls have assembled 59% and continue to accumulate cytoskeletal-associated band 3 up to at least 70% during longer periods of chase. Previous studies from this laboratory have shown that the assembly of the peripheral membrane polypeptides ankyrin, protein 4.1, and spectrin is rapidly halted by the addition of inhibitors of protein synthesis (4, 33, 40, 45, 46). These results indicate that band 3 assembly most likely occurs through the recruitment of band 3 polypeptides onto a preassembled membrane cytoskeleton. Furthermore, the pool of membrane cytoskeletal binding sites for band 3, presumably one of the peripheral membrane cytoskeletal polypeptides, eventually saturates after the addition of the inhibitor indicating that the number of binding sites has become limiting. This suggests that preassembly of certain components of the peripheral membrane cytoskeleton is a prerequisite for band 3 recruitment and binding.

Although the nature of the mechanism that controls band 3 assembly remains to be established, one possibility is that it results in or requires structural or conformational changes in band 3 prior to or upon association with the membrane cytoskeleton. For this reason, the posttranslational modifications that result in the 100- and 105-kD band 3 polypeptides observed in chicken erythroid membranes at steady state were also investigated through a variety of enzymatic digestions. Endo H treatment of band 3 immunoprecipitates from 10-d erythroid cells pulsed for 10 min with [ $^{35}$ S]methionine resulted in two polypeptides of 95 and 97 kD that undergo the addition of high-mannose chain sugars to yield the 97- and 100-kD polypeptides seen in untreated controls. Previous investigators have demonstrated similar modifications for murine erythroid band 3 (5). At present it is not known whether the 97-kD polypeptide is derived from the 95-kD polypeptide and is insensitive to endo H digestion or is synthesized *de novo*. After transport of the 97- and 100-kD band 3 polypeptides to the plasma membrane and during their association with the membrane cytoskeleton, additional posttranslational modifications occur, some of which are not endo H sensitive. Two lines of evidence suggest that the association of band 3 polypeptides with the membrane cytoskeleton results in modifications that the detergent-soluble polypeptides do not undergo. The first is that endo H digestion of detergent-soluble band 3 polypeptides after 2 h of chase still yields a discrete polypeptide of 95 kD whereas digestion of detergent-insoluble band 3 results in a diffuse pattern of polypeptides ranging from 95 to 97 kD. Secondly, the band 3 products resulting from proteinase K digestion from the outside of the cell differ extensively between the detergent-soluble and -insoluble fractions (Fig. 4, *B* and *D*). Both of these results suggest that either as a result of or as a prerequisite for associating with the membrane cytoskeleton, band 3 polypeptides undergo structural or conformational changes that are not detected in the detergent-soluble band 3 polypeptides. Previous experiments using ankyrin-depleted adult human erythrocyte membranes have shown that only 12% of the band 3 polypeptides, which is approximately the same percentage associated with the membrane cytoskeleton in adult erythrocytes (2), have the capacity to rebinding ankyrin while purified band 3 reconstituted into membrane vesicles can bind ankyrin to a greater extent (19). This result further supports the idea that band 3 polypeptides undergo structural or conformational changes as a result of or as a prerequisite for their association with the membrane cytoskeleton.

Immunoblotting analysis suggests that one factor that regulates the extent to which band 3 associates with the cytoskeleton is the availability of membrane cytoskeletal binding sites early in development. The very low levels of cytoskeletal band 3 early in development may be due to the low levels of ankyrin and protein 4.1 providing a limiting number of potential binding sites. As development proceeds, ankyrin and protein 4.1 levels increase concurrently with cytoskeletal-associated band 3 and by day 10 virtually all of the band 3 molecules are associated with the cytoskeleton. After day 10, cytoskeletal band 3 levels decline even though the steady-state levels of ankyrin and protein 4.1 continue to increase and are in apparent excess of the declining band 3 polypeptide levels. These results indicate that the extent to which band 3 associates with the cytoskeleton during development

is not regulated solely by the availability of membrane cytoskeletal binding sites but must be influenced by additional factors. Band 3 assembly onto the membrane cytoskeleton may also require a critical concentration of band 3 in the plasma membrane. Thus, the extent of assembly would be dependent upon the total amount of band 3 present at a given stage of development. The data presented in Table I indicate that increases and decreases in cytoskeletal-associated band 3 correlate with changes in the steady-state level of band 3 and are also consistent with this mechanism of assembly.

The steady state analysis further indicates that the extent of accumulation of ankyrin and protein 4.1 is not totally dependent upon the level of cytoskeletal-associated band 3. The changing ratios of ankyrin and protein 4.1 to band 3 during development suggest that these polypeptides can use alternative binding sites to assemble onto a stable membrane cytoskeleton. Previous studies with mammalian red blood cells have demonstrated that in addition to band 3, ankyrin specifically interacts with spectrin *in vitro* (2, 30), while protein 4.1 forms a ternary complex with spectrin and actin (10, 16, 43) as well as binding the cytoplasmic domain of glycophorin (1). It will be of interest to determine whether there is an avian equivalent to glycophorin which, through its interaction with protein 4.1, may mediate the attachment of the cytoskeleton to the membrane in the absence of band 3. A previous hypothesis suggested that the availability of band 3 polypeptides limits the extent to which the peripheral membrane cytoskeletal polypeptides can assemble onto the membrane cytoskeleton as well as their stability (29, 33). The results presented here indicate that band 3 does not limit the assembly of these peripheral membrane cytoskeletal polypeptides; however, the association of band 3 with the peripheral polypeptides may still result in their stabilization.

These studies have shown that the accumulation of band 3 polypeptides precedes ankyrin and protein 4.1 early in development and peaks in 10-d erythroid cells. After day 10, band 3 polypeptide levels decline by 50% and remain constant until hatching whereas ankyrin and protein 4.1 continue to increase to their maximum levels on day 18 of development. This continuous accumulation of ankyrin and protein 4.1 during development appears to be specific for these membrane cytoskeletal polypeptides inasmuch as it is not seen for other cytoskeletal polypeptides such as  $\beta$ -tubulin which declines 65% (20) or actin which declines 90% (21) over a similar period. Furthermore, the developmental fluctuations observed in the steady-state polypeptide levels of band 3, ankyrin, and protein 4.1 are not reflected in their RNA levels. The per cell amount of RNA for each of these polypeptides is highest in 5-d erythroid cells and decreases as development proceeds, although the decline in the level of ankyrin and protein 4.1 RNA is much more gradual than that seen for band 3 RNA. These results strongly suggest that the extent of accumulation of band 3, ankyrin, and protein 4.1 polypeptides early in embryogenesis is not determined primarily at the level of mRNA abundance. Previous studies have demonstrated that  $\alpha$ -spectrin,  $\beta$ -spectrin, ankyrin, and protein 4.1 turn over with a much shorter half-life in avian erythroblastosis virus transformed erythroid progenitor cells, which do not synthesize band 3, than in mature erythroid cells (44). This raises the possibility that the low levels of ankyrin and protein 4.1 polypeptides in 4-d erythroid cells result from a relatively high turnover of these polypeptides

in the absence of a stable membrane cytoskeleton. In that the RNA levels for these three polypeptides are maximal at this stage of development, it remains to be established whether a high protein turnover rate is the only factor that contributes to the low steady-state polypeptide levels or whether translational control also plays a role.

Finally, the observation that band 3 assembly is lineage and differentiation independent suggests that the environment of erythroid cells is an important factor in regulating the cytoskeletal association of band 3 polypeptides. This raises the intriguing possibility that the level of cytoskeletal-associated band 3 may be hormonally regulated. It is interesting to note that the peak of band 3 assembly on day 10 of development coincides with a peak in the levels of cAMP and protein kinase activity in chicken embryos (48). Previous studies have indicated that chicken erythroid band 3, protein 4.1 (17) and ankyrin (36) are phosphorylated. In addition, the phosphorylation of protein 4.1 reduces its affinity for spectrin (15) and may result in its association with band 3 in that spectrin and band 3 share a common binding site on protein 4.1 (37). These results suggest that the association of band 3 with the erythroid membrane cytoskeleton may be regulated at multiple levels by the phosphorylation of its constituent polypeptides.

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